Fatty acid auxotrophy in *Drosophila* larvae lacking SREBP

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Summary

SREBPs are membrane bound transcription factors that are crucial for normal lipid synthesis in animal cells. Here, we show that *Drosophila* lacking dSREBP die before the third larval instar. Mutant larvae exhibit pronounced growth defects prior to lethality, along with substantial deficits in the transcription of genes required for fatty acid synthesis. Compared to wild-type larvae, mutants contain markedly less fatty acid, although its composition is unaltered. Dietary supplementation with fatty acids rescues mutants to adulthood. The most effective fatty acid, oleate, rescues 80% of homozygotes. Rescue by dSREBP requires expression only in fat body and gut. Larvae expressing dSREBP prior to pupariation complete development and are viable as adults even when dSREBP expression is subsequently extinguished. The role, if any, of dSREBP in adults is not yet apparent. These data indicate that dSREBP deficiency renders *Drosophila* larvae auxotrophic for fatty acids.

Introduction

Mutants that lose the capacity to synthesize specific end products become dependent on external supplies. These mutants, called auxotrophs (Davis and Mingioli, 1950), have been invaluable in dissecting metabolic pathways in bacteria and yeast and to some extent in mammalian cells. To date, however, the power of fly genetics has rarely been used to explore auxotrophy in a higher eukaryote. Examples in flies include deficiencies in nucleoside and sphingolipid biosynthesis (Adachi-Yamada et al., 1999; Falk and Nash, 1974; O'Donnell et al., 2000).

In this paper, we describe mutations in flies that render them auxotrophic for fatty acids. The mutants involve a regulatory pathway centered on sterol regulatory element binding protein (SREBP). In all metazoans studied, the SREBPs are transcription factors required for synthesis of cholesterol and unsaturated fatty acids. SREBPs are synthesized as precursors of ~120 kDa containing two membrane-spanning helices (Sato et al., 1994) inserted into endoplasmic reticulum (ER) membranes. Both the transcriptionally-active NH_2-terminal domain and the COOH-terminal regulatory domain are cytoplasmic. A short loop separates the membrane-spanning helices and projects into the lumen of the ER, giving the precursor a hairpin configuration (Brown and Goldstein, 1997). In the ER, the SREBP precursor binds to an escort factor, SREBP cleavage activating protein (Scap).

In response to cellular lipid needs, the SREBP:Scap complex exits the ER and travels to the Golgi apparatus. There, it is subject to sequential cleavages by two distinct proteases, Site-1 protease (S1P) and Site-2 protease (S2P) (Brown and Goldstein, 1999). The second cleavage releases the transcriptionally active domain from the membrane, enabling it to travel to the nucleus and activate the transcription of target genes (Brown and Goldstein, 1997). In mammalian cells, the build up of cholesterol in ER membranes prevents exit of SREBP:Scap from the ER and thereby reduces lipid synthesis.

The genome of *Drosophila melanogaster* encodes a single SREBP homolog, *dSREBP* (also called *HLH106*, [Theopold et al., 1996] or *CG8522*), in contrast to vertebrates that produce three isoforms of SREBP from two loci. *Drosophila* also have genes for S1P, S2P, and Scap (Seegmiller et al., 2002). In *Drosophila* Schneider S2 cells, production of transcriptionally active nuclear dSREBP requires intact cleavage sites for S1P and S2P as well as the presence of functional Scap (Seegmiller et al., 2002). Therefore, there are substantial similarities between the insect and vertebrate pathways of lipid homeostasis. There are also significant differences. Flies cannot synthesize cholesterol de novo and the major metabolic product mediating feedback regulation of dSREBP cleavage in S2 cells is a phospholipid, phosphatidylethanolamine (Dobrosotskaya et al., 2002). Accordingly, cholesterol has no effect on processing of dSREBP (Seegmiller et al., 2002).

To determine the role of *dSREBP* in maintenance of lipid homeostasis in flies, we isolated mutations in the gene encoding dSREBP. We find that loss of *dSREBP* results in a transcriptional deficit of the genes of fatty acid synthesis, reduced fatty acid content, and larval lethality. Lethality is suppressed by supplementing the diet with fatty acids. Dietary supplementation also suppresses dSREBP cleavage and the accumulation of its target genes in wild-type larvae. Thus, mutation of a single locus causes a transcriptional deficit in mutant animals that can be overcome by nutritional supplementation. These results provide evidence, at an organismal level, that the essential function of SREBP in flies is the maintenance of fatty acid homeostasis.

Results

dSREBP is an essential gene

To obtain flies harboring deletions in the *dSREBP* open reading frame (ORF), we used transposase-mediated P element excision (Robertson et al., 1988). We screened 1200 independent excisant lines by a combination of Southern blotting and PCR analysis and identified ten lines with deletions extending into *dSREBP*. One line, deletion 189, was selected for further study (Figure 1A).

Genomic DNA from deletion 189 flies was analyzed by direct sequencing of PCR products from primers flanking the deletion. Comparison to the wild-type sequence revealed that the deletion originates at the 5′ end of the site of P element insertion. It

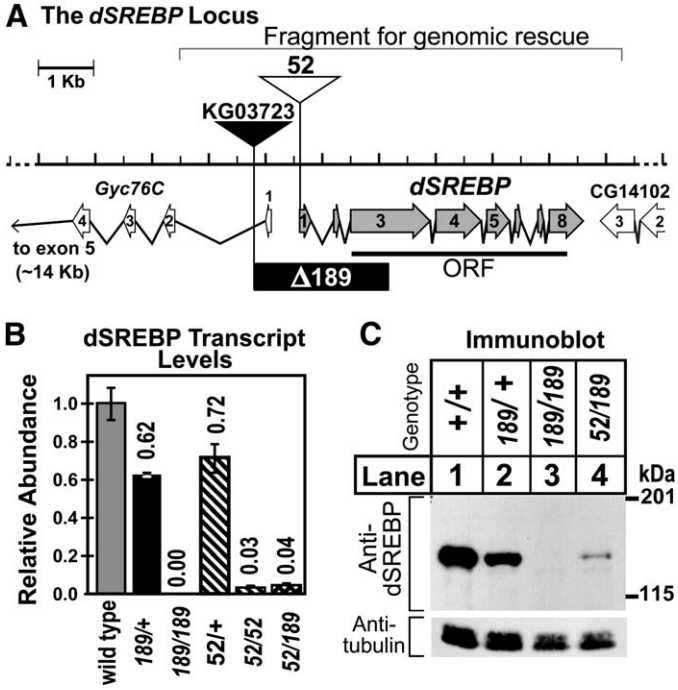

Figure 1. Characterization of *dSREBP* mutants

A) Map of the *dSREBP* locus. The *dSREBP* gene is indicated in light gray, with exons indicated by arrows pointing in the direction of transcription. The open reading frame is indicated by a heavy line (ORF). Triangles indicate sites of transposon insertion. KG03723 (black triangle) is the P element mobilized to generate $dSREBP^{189}$ (filled bar), a deletion that extends 697 bp into the ORF. $dSREBP^{52}$ (white triangle) is a piggyBac transposon insertion at bp 3 of exon 1 that disrupts *dSREBP* expression (Horn et al., 2003).

B) Quantitative analysis of dSREBP transcripts in wild-type versus mutant first instar larvae of the genotype indicated. The number above each bar indicates the relative abundance of dSREBP transcripts as compared to wild-type (= 1). Error bars represent the SD.

C) Immunoblot analysis of whole-fly lysates of the genotype indicated (30 μg/lane). The blot was probed with monoclonal antibody against the NH_2-terminal fragment of dSREBP (upper panel). The membrane was then stripped and reprobed with anti-tubulin antibody as a loading control (lower panel).

removes all transposon sequences and extends 697 nucleotides into the *dSREBP* ORF, up to amino acid 233 in exon 3. The next in-frame start codon is at amino acid 265, immediately preceding the DNA binding domain. Under standard culture conditions, balanced stocks of deletion 189 yielded few homozygous adults (0%–4% of expected) that eclose 5–8 days after their heterozygous siblings (data not shown).

In addition to disrupting the *dSREBP* gene, deletion 189 removes the predicted first exon of an adjacent gene, *Gyc76C.* Our characterization of deletion 189 homozygotes rules out potential contribution of *Gyc76C* to the observed phenotypes (see below). In order to confirm that lethality of deletion 189 homozygotes results from disruption of *dSREBP* and not from disruption of any other gene, we performed P element-mediated germline transformation (Rubin and Spradling, 1982). For the rescue construct, we used a fragment of genomic DNA including the entire *dSREBP* gene but no other coding sequences (P{*dSREBPg*}; Figure 1A). When introduced into deletion 189 homozygotes as a single copy on the second chromosome, two independent insertions of this construct completely rescued lethality and restored normal rates of development (Table S1 in the Supplemental Data available with this article online). Furthermore, these rescued flies can be maintained as stocks that are homozygous for deletion 189. Constructs expressing dSREBP cDNA also afforded efficient rescue (Table S2). Thus, only dSREBP itself is necessary to fully rescue lethality of deletion 189 mutants and therefore disruption of *dSREBP* (but not of any other sequences contained within this deletion) is lethal to flies prior to adulthood. For convenience, we will refer to the deletion 189 homozygotes as d*SREBP* mutants ($dSREBP^{189}$).

We obtained a piggyBac transposon insertion in *dSREBP* located 3 bp into exon 1 (Figure 1A). This allele, designated $dSREBP^{52}$ (Horn et al., 2003), is also substantially lethal when homozygous, as are the two alleles *in trans*. As measured by real-time RT-PCR using primers specific for exon 8 (outside the deletion; Figure 1A), transcription of *dSREBP* was profoundly deficient in both the insertion and deletion mutants (Figure 1B). In first instar $dSREBP^{52}/dSREBP^{52}$ or $dSREBP^{52}/dSREBP^{189}$ larvae, transcripts were detected at less than 5% of the wild-type level while in $dSREBP^{189}/dSREBP^{189}$ larvae, transcripts are consistently detected at less than 0.5% of wild-type levels.

Figure 1C shows an immunoblot of lysates of adult male flies using an antibody directed against the NH_2-terminal domain of dSREBP. In wild-type males (lane 1) or in $dSREBP^{189}$/+ males (lane 2), the precursor form of dSREBP is readily detected. In the rare ''escaper'' $dSREBP^{189}/dSREBP^{189}$ flies, no dSREBP precursor is detected (lane 3). In $dSREBP^{52}/dSREBP^{189}$ males, low levels of dSREBP protein are detectable (lane 4). Detection of dSREBP transcripts and protein in $dSREBP^{52}/dSREBP^{189}$ mutants indicates that low levels of transcription occur from the insertion allele. Therefore, $dSREBP^{189}$ is a null allele and $dSREBP^{52}$ is strongly hypomorphic.

For experiments where the endpoint is the rescue of lethality (or lack thereof), we present data for the null $dSREBP^{189}$ allele. For experiments exploring phenotypes other than lethality, we display data from the strongly hypomorphic $dSREBP^{52}/dSREBP^{189}$ allele combination (Figure 1C) in order to rule out contribution to these phenotypes from any potential disruption of *Gyc76C*.

dSREBP mutants fail to progress through second instar

Figure 2A shows the lethal phase for the *dSREBP* mutants. Between 1.5 and 3 days after egg laying, corresponding approximately to the first two larval instars, the frequency of *dSREBP* homozygotes in the population is near the expected for both alleles, with $dSREBP^{189}/dSREBP^{189}$ larvae showing a somewhat lower frequency than $dSREBP^{52}/dSREBP^{52}$. By 3–3.5 days, the frequency of homozygotes diminishes considerably. By 4–4.5 days, almost no homozygotes are observed. Thus, flies lacking *dSREBP* die predominantly at the time when they should have become third instar larvae. $dSREBP^{52}/dSREBP^{189}$ larvae die at this same larval stage (Figure S1A), indicating that death at this time point occurs solely owing to mutations in *dSREBP*. Comparison of $dSREBP^{189}/dSREBP^{189}$ larvae to their $dSREBP^{189}$/+ siblings showed that failure to reach third instar in homozygotes correlated with a profound growth defect during the second larval instar (Figure 2B). The few homozygotes that do progress to third instar (as determined by anterior spiracle morphology) are typically undersized (data not shown). We observed comparable results with $dSREBP^{52}/dSREBP^{52}$ and $dSREBP^{52}/dSREBP^{189}$ larvae (data not shown).

To determine if dSREBP activity is required in adult flies, we employed the P{Switch} system to control expression of

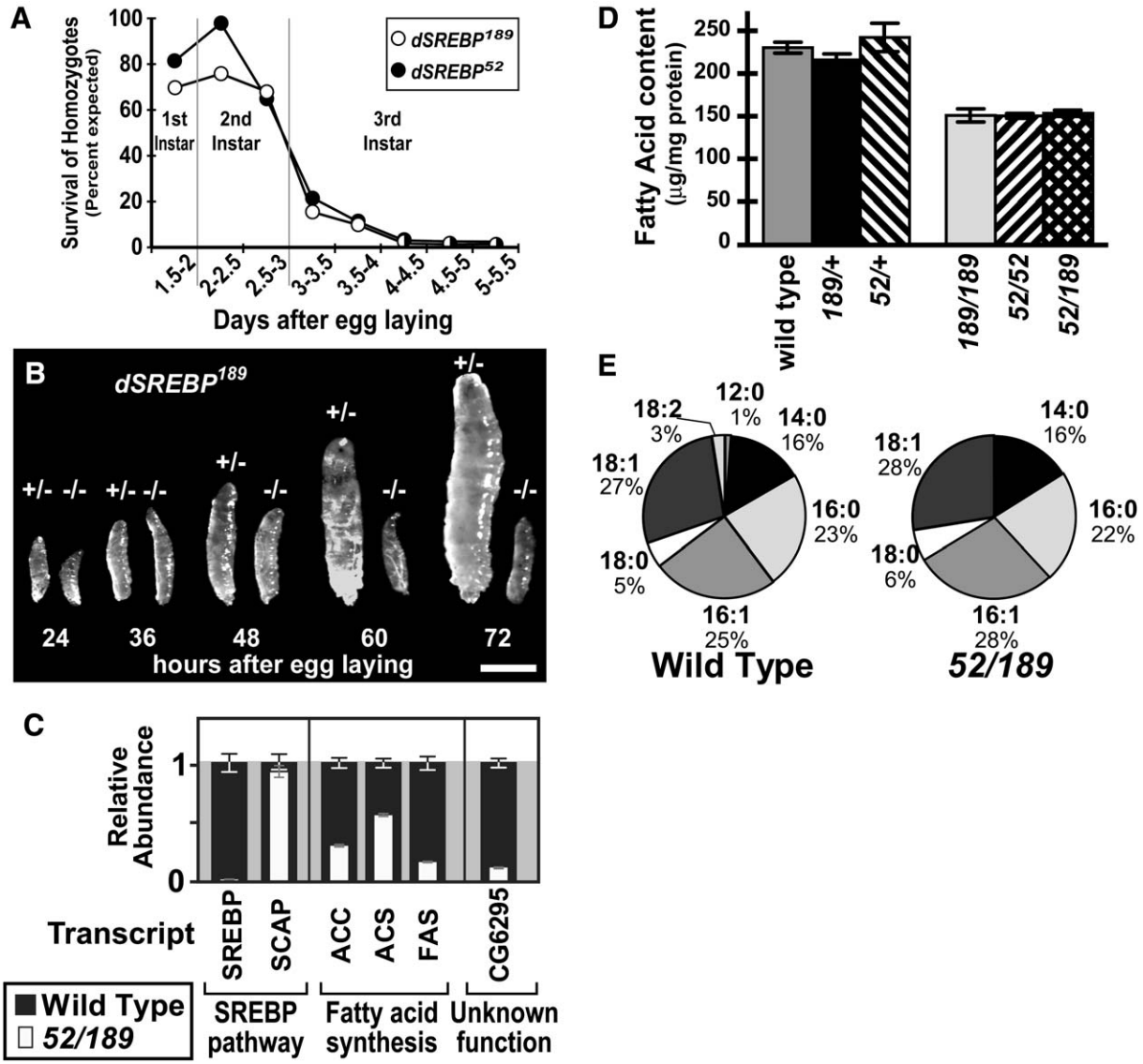

Figure 2. Phenotypes of *dSREBP* homozygous mutants

A) Larvae from *dSREBP*^{189} */TM3, Actin-GFP, Ser* or *dSREBP*^{52}*/TM3, Actin-GFP*, *Ser* stocks were collected at each time point, and the ratio of homozygous to heterozygous larvae was determined as described in Experimental Procedures. A mean of 500 larvae were scored at each time point (range = 367–821).

B) Comparison of size differences between *dSREBP*^{189} heterozygous (+/−) and homozygous (−/−) larvae. Embryos were plated as described for the lethal phase assays, and larvae photographed at the indicated time points. The scale bar represents 1 mm.

C) Quantitative analysis of transcripts. The indicated transcripts were measured in wild-type and *dSREBP*^{52}*/dSREBP*^{189} first instar larvae. Error bars represent the SD.

D) Total fatty acid content of first instar larvae (μg fatty acid/mg protein). Triplicate samples were measured for each genotype and the mean plotted. Error bars represent the standard error of the mean. Samples were prepared and analyzed as described in Experimental Procedures. The *dSREBP* homozygous and transheterozygous samples differ significantly from the control samples (wild-type and heterozygotes) at $p < 0.001$ by Student's two-tailed t test.

E) Fatty acid composition of wild-type and *dSREBP*^{52}*/dSREBP*^{189} first instar larvae as % of total for all species detected at >0.1% of total. The composition observed for *dSREBP*^{189}*/dSREBP*^{189} and *dSREBP*^{52}*/dSREBP*^{52} mutants do not differ significantly from the transheterozygotes shown (Table S3). The fatty acid composition does not differ significantly among any of the genotypes tested as determined by a χ^2 test of independence ($p > 0.8$ for each genotype).

dSREBP in a temporal manner. In this variation of the GAL4 enhancer trap system, the yeast GAL4 transcription factor is fused with a progesterone receptor (PR) ligand binding domain. The transcription of genes under the control of the yeast upstream activating sequence (UAS) then depends on the presence of PR ligands such as RU486 (Roman et al., 2001).

We used P{Switch} line $S_1$106 (Roman et al., 2001) to drive expression of dSREBP in *dSREBP*^{189}*/dSREBP*^{189} larvae. In the presence of RU486, these animals survived to pupariation and emerged as adults (Table S2). The rescued homozygous adults survived at least two weeks in the absence of RU486 (data not shown). In the absence of RU486, no dSREBP is detectable in the rescued adults (Figure S2). Thus, dSREBP activity is essential during larval life but is not strictly required in adults. The bulk of our studies therefore focused on dSREBP function in larvae.

We had previously shown that when dSREBP activity in *Drosophila* S2 cells was diminished by RNAi treatment, de novo synthesis of fatty acids fell 4-fold (Seegmiller et al., 2002). We had also identified acetyl coenzyme A (Ac CoA) carboxylase (ACC), Ac CoA synthase (ACS), and fatty acid synthase (FAS)

genes as highly regulated dSREBP targets. *dSREBP* mutant larvae of each genotype showed deficits in the transcription of these genes in first instar, prior to the onset of growth arrest. Figure 2C shows data from $dSREBP^{52}/dSREBP^{189}$ larvae as compared to wild-type. Additionally, in studies to be reported elsewhere, we identified a gene of undetermined function, *CG6295*, as a potential dSREBP target. Its transcription was also deficient in mutant larvae (Figure 2C).

We measured the fatty acid content of first instar larvae and found that *dSREBP* mutants contained significantly less total fatty acid than wild-type or heterozygous larvae (Figure 2D). However, the relative abundance of various fatty acid species did not differ significantly among these animals (Figure 2E, Table S3).

Expression required in fat body and midgut

The piggyBac transposon in the $dSREBP^{52}$ allele encodes a GAL4 enhancer trap. When used to drive a UAS-GFP construct, it permitted determination of where *dSREBP* is transcribed. In larvae, the *dSREBP* promoter is active in fat body, midgut, and oenocytes. However, dSREBP requires transport and cleavage to produce active transcription factor from the membrane bound precursor. To determine in which tissue(s) dSREBP is not only expressed but active, we designed a reporter system to follow dSREBP cleavage in living animals. We replaced the transcription factor domain of the genomic rescue construct (pP{*dSREBPg*}) with GAL4-VP16 to make pP{*GAL4-dSREBPg*}(Figure 3A). This construct is transcribed under control of the native *dSREBP* promoter(s) and the resulting chimeric protein (GAL4-dSREBP) is subject to the same physiologically-regulated proteolytic processing as wild-type dSREBP (Figure S3). When used to drive expression of full-length dSREBP cDNA, GAL4-dSREBP afforded complete rescue of *dSREBP* mutants (Table S4).

We used this construct to drive expression of a UAS-GFP construct in transgenic animals and identify tissues in which dSREBP is active (Figure 3). No green fluorescence was seen in animals harboring either transgene alone (Figure S4). Thus, green fluorescence is an indicator of where dSREBP is expressed and cleaved in these animals.

Flies harboring both the P{*GAL4-dSREBPg*}driver and the P{UAS-GFP} responder transgenes in a wild-type background showed activity throughout larval development (Figure 3B). Substantial activity was seen in fat body, midgut, and in oenocytes of larvae (Figures 3B and 3C) and in the corpus allatum of the ring gland (3D). Differences were apparent between larval and adult patterns of expression in fat body and midgut. In larvae, these tissues were major sites of dSREBP activity (Figures 3B and 3C). Adults showed weak or no expression in fat body (Figure 3E), though activity was observed in discrete patches of the midgut (Figure 3H). Oenocytes continued to show intense activity in adults (Figures 3E–3G). In males, the ejaculatory bulb also showed intense fluorescence (Figure 3H) as did the proventriculus (cardia) in both sexes (Figure 3I and data not shown [males]).

To explore further the tissues in which activity of dSREBP is required, we placed a full-length dSREBP cDNA under control of yeast UAS. We performed experiments using various GAL4 enhancer trap and promoter fusion lines to drive its expression in spatially restricted domains during larval life. Table 1 correlates the expression of these various drivers (in tissues where dSREBP is active) with their ability to rescue $dSREBP^{189}$ mutants. The expression pattern of the GAL4-dSREBP driver is included as a reference for the domains in which dSREBP activity is normally detected.

The S_1106 driver, which rescues *dSREBP* null animals, is expressed only in the midgut and fat body. These are therefore the tissues where dSREBP activity is *sufficient*. Activity in the oenocytes and ring gland is not required for survival. We further attempted to dissect whether expression in either the fat body or the midgut alone is necessary. For this, we used the DcG-GAL4 and the 6450 driver transgenes. The DcG-GAL4 driver, which rescues only weakly, is expressed strongly in the fat body but not in any part of the gut. Therefore, expression in the gut is *necessary* for full viability. The 6450 driver, which does not rescue at all, is expressed strongly in the gut but *not* in the fat body. Therefore, expression in the fat body is also *necessary* for viability. Expression in other tissues does not afford rescue if dSREBP is not also expressed in fat body and (optimally) in midgut. We conclude that dSREBP function is essential in the fat body and midgut.

Failure of 6450 and DcG-GAL4 to rescue did not simply result from weak expression of GAL4. A UAS-GFP reporter transgene revealed strong fluorescence in the midgut and fat body, respectively, with these nonrescuing drivers (Figures S5B and S5C).

Lethality rescued by dietary supplementation

We hypothesized that the lethality of *dSREBP* mutants might result from a lipid deficiency secondary to the transcriptional deficit of genes needed for lipid synthesis such as FAS. To test this, we supplemented fly culture media with a number of different lipids and evaluated their ability to rescue *dSREBP* mutants. The survival of $dSREBP^{189}/dSREBP^{189}$ flies to adulthood was markedly improved in the presence of soy lipids (also called "lecithin") and increased with increasing concentration (Figure 4A). The homozygous adults that emerged from supplemented cultures were indistinguishable in mass from their heterozygous siblings (Figure 4B), and were morphologically normal (Figure 4C). The results for $dSREBP^{189}/dSREBP^{189}$ mutants are shown since they are null for dSREBP expression and thus afford a more rigorous test of the ability of soy lipid supplementation to supplant all the essential functions of dSREBP than do the hypomorphic $dSREBP^{52}$ mutants. Transheterozygous $dSREBP^{52}/dSREBP^{189}$ animals also show increased survival on supplemented medium (Figure S1B). Supplementation of the larval diet with soy lipids thus restores nearly normal growth to *dSREBP* mutants.

Supplementation suppresses cleavage of dSREBP

If soy lipids provide an end product of dSREBP activation, then that product should suppress cleavage of dSREBP in a manner analogous to the action of cholesterol in mammalian cells. In the presence of increasing concentrations of soy lipids, we observed diminished accumulation of the nuclear form of dSREBP (Figure 5A). Suppression was also observed with the GAL4-*dSREBPg*/UAS-GFP reporter system. Larvae reared on unsupplemented medium (Figure 5B, upper left) showed much greater fluorescence relative to siblings reared on medium supplemented with 9% soy lipids (lower left).

This difference results from proteolytic regulation of dSREBP activity. By contrast to the membrane bound product of the P{GAL4-dSREBPg}construct, the GAL4 in the $dSREBP^{52}$ enhancer trap line is expressed as a soluble protein that does not require cleavage for transcriptional activity. When we used

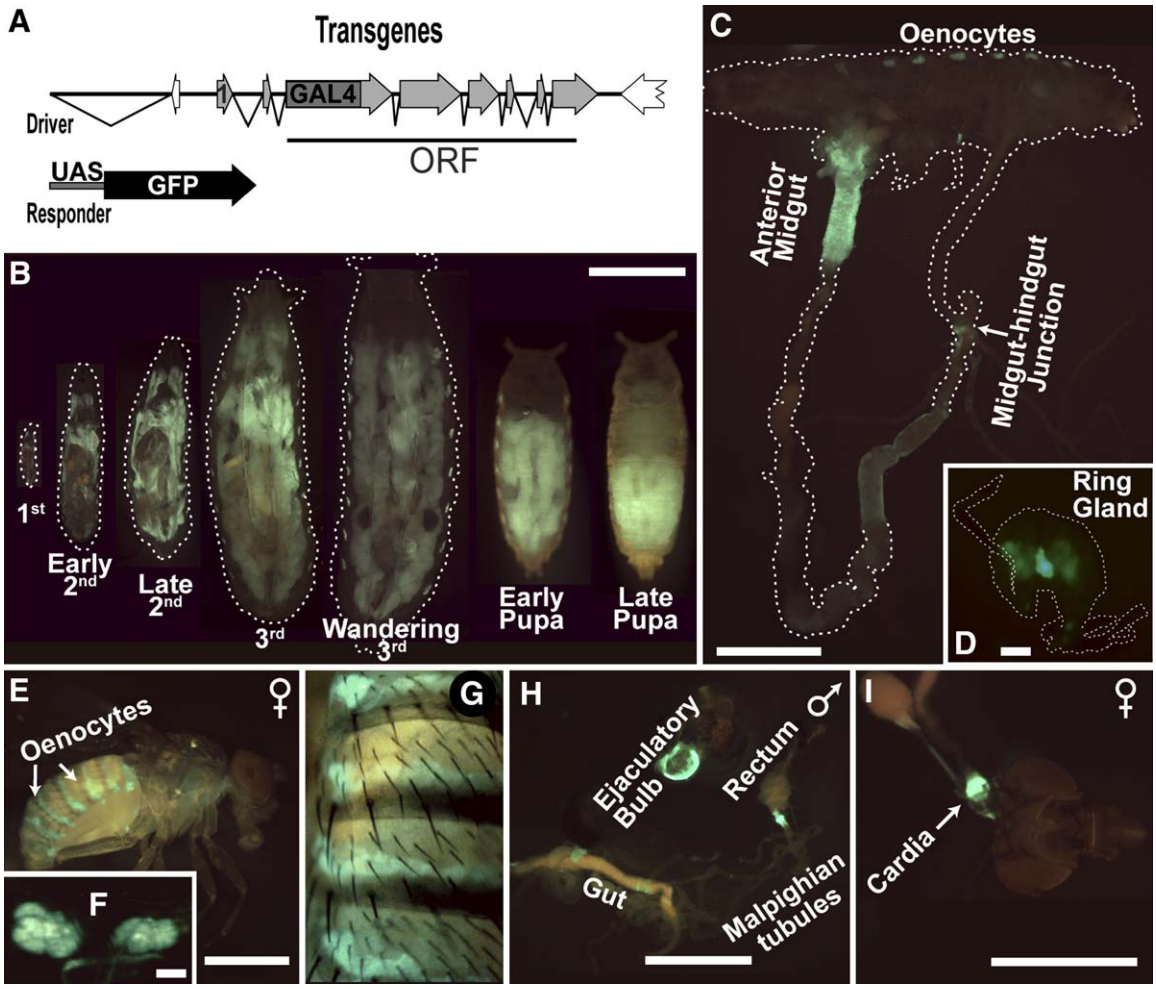

Figure 3. Localization of dSREBP cleavage

A) A binary reporter system for dSREBP activity. The transcription factor domain of pP{*dSREBPg*} was replaced by a GAL4-VP16 transcription factor to generate pP{*GAL4-dSREBPg*}. Animals transgenic for both P{*GAL4-dSREBPg*} and P{UAS-GFP} were examined for spatial localization of GFP fluorescence.

B) Dorsal views of larvae and early pupae. At all larval stages, fluorescence is detected in fat body, midgut, and oenocytes. The contents of the gut autofluoresce (Figure S4). In late pupae, fluorescence can be detected throughout the animal.

C) Dissection of a third instar larva shows two domains of dSREBP activity in the midgut: (1) a strong signal in the midgut and (2) weaker signal in a region encompassing the posterior portion of the midgut. In this preparation, the fat body has been removed so that the oenocytes are more distinctly visible.

D) GAL4-dSREBP activity in the ring gland of a wandering third instar larva. The corpus allatum shows intense fluorescence. Fluorescence is also detectable in the lateral portions of the gland.

E) In adults of either sex, signal can be detected in oenocytes (female shown).

F) Oenocytes at the posterior margin of an abdominal tergite.

G) Dorsal view of a female abdomen showing GFP fluorescence in bands of oenocytes.

H) In males, strong GFP fluorescence can be detected in the ejaculatory bulb. In either sex, a region anterior to the rectum along with isolated regions of the midgut also show activity (male shown).

I) The cardia shows intense fluorescence in both sexes (female shown).

All scale bars represent 1 mm, except for (**D**) and (**F**), for which they represent 0.05 mm. Dashed lines denote the extent of larval tissues.

this insertion to drive UAS-GFP, strong fluorescence was observed in fat body and midgut on both unsupplemented and 9% soy lipids media (Figure 5B, right panels). Thus, transcription from this promoter is not responsive to the diet, while activation of dSREBP is responsive.

Suppression of dSREBP cleavage leads to reduced accumulation of transcripts of target genes. We compared the abundance of transcripts in wild-type first instar larvae cultured in the presence or absence of 9% soy lipids. In larvae reared on supplemented medium, transcript abundance for dSREBP, ACC, ACS, and FAS were reduced as compared to larvae on unsupplemented medium (Figure 5C). By contrast, transcripts for Scap (a gene also unaffected in the *dSREBP* mutant animals [Figure 2C]) were unchanged. In $dSREBP^{189}/dSREBP^{189}$ or $dSREBP^{52}/dSREBP^{189}$ larvae, the low levels of these transcripts were not further reduced in the presence of soy lipids (data not shown). This indicates that the transcriptional changes depend on dSREBP. Transcription of known and putative target genes in mutant larvae was not restored to wild-type levels following lipid supplementation, indicating that the reduced abundance of these transcripts in *dSREBP* mutant animals is not a consequence of end product depletion.

Table 1. Domains of GAL4 expression that rescue *dSREBP* mutants

	Rescue (%)		Midgut	Fat body	Oenocytes	Ring gland	Other
P{GAL4-*dSREBPg*}	Yes	(108)	+	+	+	+	−
$S_1$106	Yes	(91)	+	+	−	−	−
6487	Yes	(65)	+	+	+	−	+
DcG-GAL4	Weak	(8)	−	+	−	−	+
6450	No	(0)	+	−	−	−	+

Multiple lines harboring GAL4 drivers with distinct patterns of tissue expression were used to drive UAS-dSREBP in a *dSREBP*^{189}*/TM6 Tb, Hu, e* background. The ability of each GAL4 driver to rescue *dSREBP*^{189} homozygous larvae to adulthood was determined as described in Experimental Procedures (P{GAL4-*dSREBPg*}, n = 88; $S_1$106, n = 464; 6487, n = 457; DcG-GAL4, n = 242; 6450, n = 217). The expression pattern of each driver was determined by crossing the GAL4 driver lines with a line harboring a UAS-GFP reporter. The presence (+) or absence (−) of GFP fluorescence in the five domains where dSREBP is active in wild type larvae (see Figure 3) was determined by dissection of late first, second, and third instar larvae.

dSREBP mutants are fatty acid auxotrophs

Soy lipid extract is largely comprised of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol in approximately equal proportions. The concentration of this required for rescue of *dSREBP* mutants was strikingly high (maximal at 9%). Since hydrolysis of phospholipids produces free fatty acids, we tested whether any of the major fatty acids (those comprising ≥ 1% of the total fatty acid of wild-type flies) could restore growth (Table 2). The only such species not tested was C18:2 owing to its susceptibility to oxidation. The fatty acids all rescued *dSREBP*^{189} homozygous animals at much lower concentrations than did soy lipids (predominantly phospholipids) or synthetic triglyceride. For example, medium supplemented with 0.15% C18:1 afforded 80% survival of homozygotes compared to less than 60% survival on 9% soy lipids. Comparable results were observed with *dSREBP*^{52}*/dSREBP*^{189} larvae (Table S5).

The data do not permit us to account for the differing efficiencies with which the various fatty acids rescue mutant larvae. This may reflect differences in the delivery of the various fatty acid species or in their metabolic fates when supplied exogenously (Keith, 1967).

Discussion

SREBP in *Drosophila* physiology

We report that loss of dSREBP activity is lethal during larval development in *Drosophila melanogaster*. Almost all homozygous mutants die by the end of the second instar (Figure 2A). This lethality occurs solely as a result of the loss of dSREBP function. *dSREBP*^{189} homozygotes are rescued by expressing a cDNA, a genomic fragment, or by dietary supplementation. Such treatments also rescue *dSREBP*^{52}*/dSREBP*^{189} transheterozygotes; loss of exon 1 of *Gyc76C* does not contribute to the observed lethality. Dispensability of "exon 1" accords with the observation of Ayoob et al. (2004) who identified a viable line, l(3)L0909-a, that harbored a P element insertion in exon 1 of *Gyc76C*. When we used the P{Switch} system to express active dSREBP only during larval life, this rescued lethality (Table S2). Once mutants reached adulthood, dSREBP was not strictly required for viability (Figure S2).

Multiple lines of evidence demonstrate that dSREBP's essential role is the transcription of genes needed for fatty acid synthesis and uptake: (1) In larvae, dSREBP activity is readily observed in tissues involved in lipid uptake (midgut) and synthesis (fat body and oenocytes) (Figures 3B and 3C). (2) Homozygous mutant larvae are rescued by expressing dSREBP in fat body and gut (Table 1). (3) Mutant larvae show reduced levels of transcripts for fatty acid synthetic genes (e.g. ACC, ACS, and FAS; Figure 2C). This deficit is not reversed by lipid supplementation (data not shown), indicating that it results from lack of nuclear dSREBP and not as a secondary consequence of end product deficiency. (4) Mutant larvae contain markedly less total fatty acid than heterozygotes or wild-type larvae (Figure 2D). (5) In a classic end product-mediated feedback mechanism, dSREBP

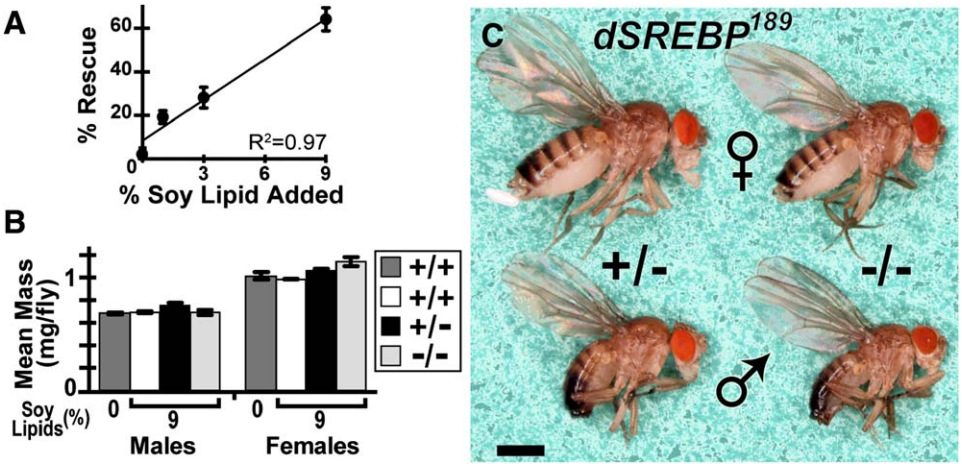

Figure 4. Soy lipid extract rescues *dSREBP*^{189} mutant animals to adulthood

A) Embryos were seeded into vials containing either regular cornmeal-molasses-agar medium or medium supplemented with increasing concentrations of soy lipid extract. Emerging adults were scored for their *dSREBP* genotype and the ratio of homozygotes/heterozygotes was used to calculate the survival of homozygotes to adulthood as a percentage of the expected ratio (0.5 = 100%). At concentrations greater than 9%, the soy lipids rendered the medium unable to support even wild-type flies due to its altered consistency.

B) Mass of *dSREBP*^{189} heterozygous (+/−) and homozygous (−/−) adults reared on medium supplemented with 9% soy lipid as compared to wild-type (+/+) adults reared on medium with or without supplementation. Mean mass was calculated for three replicates of ten adults for each condition. Error bars indicate standard error of the mean.

C) *dSREBP*^{189} heterozygous (+/−) or homozygous (−/−) adults were raised on medium supplemented with 9% soy lipid extract.

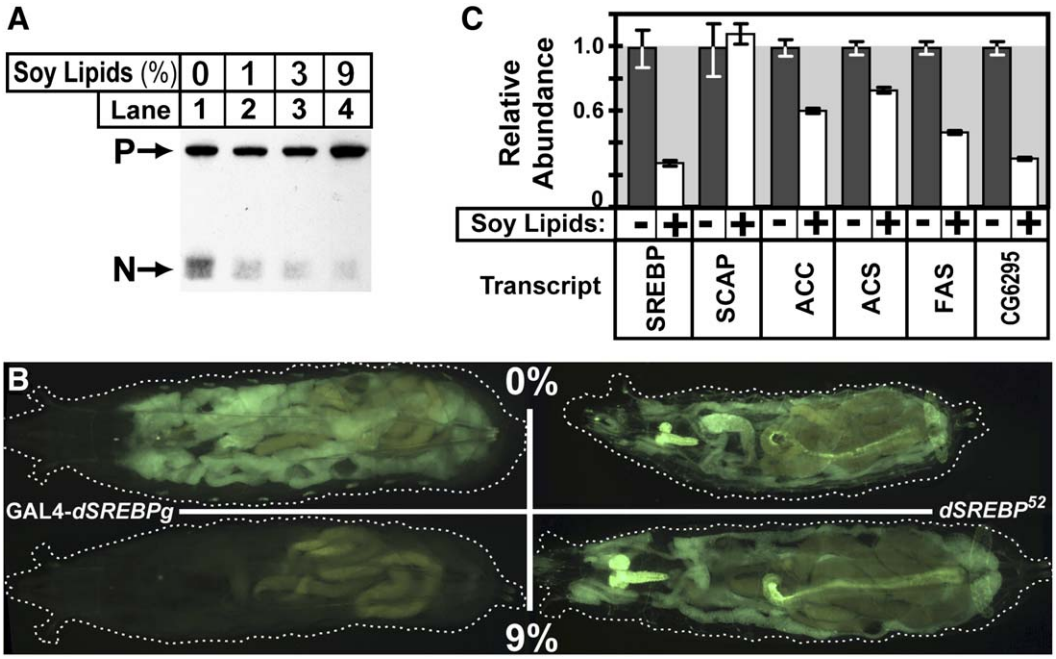

Figure 5. Supplementation with soy lipid extract suppresses dSREBP cleavage in wild-type larvae

A) Third instar larvae raised on semidefined medium supplemented with increasing concentrations of soy lipid extract were harvested and whole-larva lysates were prepared. Samples (60 μg) were analyzed by immunoblotting with anti-dSREBP. P; membrane bound dSREBP precursor. N; nuclear form.

B) Wild-type larvae carrying UAS-GFP and either P{*GAL4-dSREBPg*} (left panels) or, as a control, the *dSREBP*^{52} insertion (right panels) are shown. Larvae were cultured on either unsupplemented cornmeal-molasses-agar medium (0%) or the same medium supplemented with 9% soy lipid extract (9%). In larvae carrying P{GAL4- *dSREBPg*}, soy lipid supplementation suppresses dSREBP activity. Suppression is not seen in larvae expressing GAL4 under control of the *dSREBP* promoter. The *dSREBP*^{52} piggyBac insertion also encodes an enhanced yellow fluorescent protein (EYFP) marker that is expressed in brain and hindgut (Figure 5B, right panels; see also Figure S5A). Dashed lines denote the extent of larval bodies.

C) Quantitative analysis of transcripts of known or putative dSREBP target genes in wild-type first instar larvae raised on semidefined medium (–) or medium supplemented with 9% soy lipid extract (+). Error bars represent the SD.

cleavage in growing larvae is strongly suppressed by dietary lipids (Figure 5). (6) Feeding extra fatty acids rescues lethality in *dSREBP* mutants (Table 2). Homozygous mutants rescued by supplementation are indistinguishable from their heterozygous siblings in mass and morphology (Figures 4B and 4C).

Wild-type flies can develop on defined medium lacking all lipids save cholesterol (Sang, 1956) and can therefore synthesize all fatty acids required for growth. *dSREBP* mutant larvae, however, are unable to grow even on regular cornmeal-molasses-agar unless supplemented with fatty acids. Therefore, flies lacking *dSREBP* are fatty acid auxotrophs and an important role of *dSREBP* in *Drosophila* physiology is the maintenance of fatty acid prototrophy.

Furthermore, our data indicate that regulation of dSREBP activity enables the organism to adjust the level of de novo lipid synthesis in response to the supply of lipids in the diet (Figure 5). Thus, the growing larva can allocate resources efficiently between the syntheses of various macromolecules in response to its environment. SREBPs have been shown to similarly regulate cholesterol synthesis in the liver in mice and hamsters (Brown and Goldstein, 1997). The benefits of balancing endogenous synthesis with dietary input and lipid demand likely provide the selective pressure for conservation of the SREBP pathway in evolution.

In our studies, we identified multiple genes involved in de novo fatty acid synthesis as dSREBP targets (Figures 2C and 5C). We did not detect changes in transcript abundance for genes involved in the elongation or desaturation of fatty acids (data not shown). This differs from mouse liver, where manipulation of the SREBP pathway causes transcriptional changes leading to altered fatty acid composition (Shimomura et al., 1998). In *dSREBP* mutants, we observed only a global deficit in the fatty acid content but no change in the relative abundance of the various species (Figures 2D and 2E). In addition, dietary supplementation with any of the major fatty acids of flies served to compensate for lack of dSREBP, albeit with varying efficiency (Table 2).

Table 2. Rescue of *dSREBP*^{189} homozygotes by dietary supplementation

Compound	Percent of expected	SEM	Concentration %	Number of Trials
No additions	4.2	0.5	n/a	171
C12:0	23.5	3.9	0.075	10
Tripalmitin	28.7	11.7	2	7
C16:1	27.3	3.7	0.15	12
C16:0	51.5	4.3	0.6	52
Soy lipids	53.1	3.9	9	50
C18:0	56.9	14.0	1.2	5
C14:0	67.5	9.2	0.075	9
C18:1	80.3	11.9	0.15	9

Multiple concentrations were tested for each compound (soy lipids: 1%, 3%, and 9%; tripalmitin: 0.6%, 2%, and 6%; sodium salts of fatty acids 0.075%, 0.15%, 0.3%, and 0.6%). For C18:0, an additional experiment with 1.2% was performed. The table reports the maximal rescue obtained with each compound tested and the concentration at which this rescue was obtained. At concentrations lower or higher than shown, rescue was less robust (except for 18:0, for which the highest concentration tested produced maximum rescue). For data from *dSREBP*^{52}/*dSREBP*^{189} transheterozygotes, see Table S5. The mean number of animals in each trial is 53.

These data indicate that the mechanisms necessary for interconversion among various species of fatty acids continue to function in the absence of dSREBP-mediated transcription.

Activation of the SREBP pathway in mammals results in the preferential production of oleate (C18:1). This may reflect the need for a substrate for the esterification and storage of the other major product of the SREBP pathway, cholesterol (Repa et al., 2000; Shimomura et al., 1998). In *Drosophila,* the SREBP pathway is not involved in cholesterol synthesis (Seegmiller et al., 2002) and this distinction may underlie the observed differences in fatty acid production between mammals and *Drosophila*.

The reduced fatty acid content of dSREBP mutant larvae is unlikely to result from a selective deficit in a particular class of lipids. An inference may be drawn by comparing the abundance of myristate (C14:0) and oleate (C18:1). Myristate is relatively enriched in di- and tri-glycerides, while oleate is enriched in phospholipids (de Renobales and Blomquist, 1984). The lack of change in the relative abundance of C14:0 and C18:1 suggests a coordinate decrease in the production of both classes of lipid (Table S3).

Mechanisms of lethality

Lethality of *dSREBP* mutant larvae occurs following growth arrest at the end of the second larval instar (Figure 2B), a time when wild-type larvae begin to increase enormously in mass (Church and Robertson, 1966). Rapid growth undoubtedly places a great demand on lipid metabolism. Growing larvae must make additional cell membrane to accommodate increasing cell size. *Drosophila*, like other insects, must achieve a critical mass in order to complete development (Nijhout, 2003), termed the threshold size for metamorphosis (~0.45 mg during second instar) (Zhou et al., 2004). Inability to carry out this task may determine the timing of growth arrest and lethality. How flies monitor mass is unknown, but recent work demonstrates a key role for the prothoracic gland (Mirth et al., 2005).

Larvae reared on non-nutritive agar fail to grow and die within 2-3 days after hatching (Galloni and Edgar, 1999). Loss of *dSREBP* mimics features of starvation. For example, homozygotes fail to grow to normal size under standard culture conditions, remaining about the size of first instar larvae (Figure 2B). When wild-type larvae are starved for nutrients that must be acquired exogenously, such as certain amino acids, choline and cholesterol, pyrimidines, or vitamins, growth is arrested but the larvae can survive for an extended period. Transfer to complete medium within 6–8 days permits these starved animals to finish development (Britton and Edgar, 1998). While flies have mechanisms such as arrested growth and delayed development for coping with deficits in exogenous nutrients, these mechanisms apparently do not respond to a deficit in nutrients that are typically supplied endogenously in wild-type animals.

Death of *dSREBP* homozygotes prior to second to third instar transition may in part reflect a failure to achieve a critical mass of neutral lipid stores in fat body. Regulation of larval growth by fat body has been demonstrated previously (Britton et al., 2002; Colombani et al., 2003; Colombani et al., 2005).

A third possible reason for the observed lethality in *dSREBP* mutants is that dSREBP may be directly required for the synthesis of a specific signaling molecule that controls growth in an endocrine fashion. The present data do not permit us to distinguish conclusively between these mechanisms. We consider an endocrine mechanism the least likely, however, owing to the variety of different fatty acids that can rescue *dSREBP* mutants.

The lack of specificity in the fatty acid requirement contrasts with our previous observations in Schneider S2 cells. There, we observed a specific requirement for palmitate (C16:0) in the regulation of dSREBP cleavage. This specificity reflected the need for palmitate as a precursor for the head group of phosphatidylethanolamine (Dobrosotskaya et al., 2002). In the present study, we added additional lipids to regular cornmeal-molasses-agar medium that already contained lipids from yeast and corn. This may explain the relaxed specificity of the fatty acids required. Indeed, in cultured S2 cells, addition of exogenous ethanolamine to the medium relaxed the specific requirement for palmitate (Dobrosotskaya et al., 2002).

SREBP in the midgut

In addition to tissues involved in de novo lipid synthesis (fat body and oenocytes), dSREBP activity is also required in a tissue that is predominantly associated with nutrient digestion and absorption (midgut). Free fatty acids rescue *dSREBP* mutants at a much lower concentration than needed when fatty acids are supplied as phospholipids or triglycerides. dSREBP activity may be needed for the animal to generate absorbable free fatty acids from phospholipids and/or triglycerides. Interestingly, we observe that a major, previously unrecognized, transcriptional target of dSREBP, *CG6295* (Figure 5C), is highly similar in predicted amino acid sequence to mammalian pancreatic lipases and contains a conserved catalytic triad.

Experimental Procedures

Genetic strains

All marker mutations and balancer chromosomes are described and referenced by the FlyBase Consortium (2003). Crosses were carried out at 25°C in vials containing freshly yeasted cornmeal-molasses-agar (1 liter of cornmeal-molasses medium contains 60 g cornmeal, 15 g dry yeast, 80 ml unsulphured molasses and 12 g agar) except where noted. Oregon-R flies served as wild-type. P element transposon insertion line KG03723 and GAL4 line 6487 and 6450 were obtained from the Bloomington *Drosophila* stock center. PiggyBac transposon insertion line $dSREBP^{52}$ was provided by Ernst Wimmer (Horn et al., 2003). This chromosome also harbored an unrelated pupal lethal mutation that was removed by recombination with wild-type. The resulting allele was fully rescuable.

The P{UAS-dSREBP} and P{*GAL4-dSREBPg*} transgene insertions are on the second chromosome. These stocks were created by standard germline transformation techniques (Rubin and Spradling, 1982). S_1106 was a gift of Ron Davis (Baylor, Houston). DcG-GAL4 was provided by Jon Graff and J. Suh (U.T. Southwestern).

Buffers

Buffer A is 10 mM HEPES-NaOH (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, and 1 mM EGTA. Buffer F is125 mM Tris-HCl (pH 6.8), 8 M Urea, and 5% SDS.

Monoclonal antibodies

IgG-3B2, against the amino-terminal domain of dSREBP was described previously (Seegmiller et al., 2002). IgG-611B-1 against acetylated tubulin was obtained from Sigma (St. Louis).

Plasmids

p{UAST-dSREBP}

Full-length *dSREBP* cDNA was amplified by PCR with the addition of EcoR1-Xba1 linkers. The resulting fragment was digested and cloned into the EcoR1-Xba1 sites of pUAST (Brand and Perrimon, 1993).

pP{*dSREBPg*}
An 8.7 kb genomic fragment (containing the entire *dSREBP* gene, 2.9 kb upstream and 0.7 kb downstream) was amplified by PCR using the High-Fidelity PCR System (Roche). The forward primer used for amplification was 5′-CG TCTAGACGCATGCTCCCAGAGATGGCACTTTGG –3′ and the reverse primer was 5′-GCTCTAGACACATGTCATCACTGTCAGCGGGATACC-3′. Xba1 linkers were added during amplification and the resultant fragment was ligated into pCaSpeR-4 (Thummel and Pirotta, 1992) to obtain pP{*dSREBPg*}. The open reading frame was sequenced in its entirety.

pP{GAL4-*dSREBPg*}
Restriction sites for Asc1 and Fse1 were inserted into pP{*dSREBPg*} at the beginning of the ORF (Asc1, inserted immediately after aa3) and immediately following the bHLH region (Fse1, inserted immediately preceding aa. 362). The primers used for insertion of the Asc1 site were 5′-GCAGCATTCGCAA TGGACACGGCGCGCCTGAACTTAATAGACGCT-3′ and its reverse complement. Primers used for insertion of the Fse1 site were 5′-GCGACGGCTCCA AGGTGAAGGCCGGCCTTCAGCTGGGCACTCGGC-3′ and its reverse complement. The sites were inserted individually into pP{*dSREBPg*}. Nar1 (for the Asc1 site) or Nar1-Nhe1 fragments (for the Fse1 site) were excised out of the resultant vector and then subcloned together into Nar1-Nhe1 digested pP{*dSREBPg*}. The resultant vector pP{*dSREBPg*/AF} was sequenced in the regions that had been subject to PCR. In order to generate pP{GAL4-SREBP}, a cDNA fragment encoding a fusion of the GAL4 DNA binding domain fused to the VP16 transactivation domain was amplified by PCR from pMGstV (a gift from Thomas Sudhof, UT Southwestern). Asc1 and Fse1 linkers were added during amplification. This fragment was then ligated into pP{*dSREBPg*/AF}.

Lethal phase assays
Embryos from *dSREBP* ^{189} /TM3, *Actin-GFP, Ser* or *dSREBP* ^{52}/TM3, *Actin-GFP, Ser* stocks or from a cross between the two were plated on 60 mm dishes (1 dish/time point) containing semidefined medium (Backhaus et al., 1984) at a density of 20 mg embryos/plate. At the indicated time, all larvae were washed off the plates, separated from the food by floatation on 2-3 M NaCl, and scored based on fluorescence detection of actin-driven GFP. Survival of homozygotes is plotted as a percentage of the expected ratio of homozygotes to heterozygotes (0.5 = 100%).

Whole-fly Lysis
Fifteen adult males or third instar larvae of the indicated genotype were homogenized in buffer F supplemented with a cocktail of protease inhibitors (Seegmiller et al., 2002). Homogenates were cleared by centrifugation at 20,000 g for 10 min. The indicated amount of the lysates were electrophoresed, transferred to a nitrocellulose membrane and probed with IgG-3B2 at 2 μg/ml.

Transgenic rescue
dSREBP cDNA rescue
The GAL4 drivers were crossed into a *dSREBP* ^{189} background to generate *w*^{1118};P{*w*$^{+}$,*GAL4*}/ P{*w*$^{+}$,*GAL4*}; *dSREBP* ^{189} */TM6B, Tb Hu e* or *w*^{1118};P{*w*$^{+}$,*GAL4*}/ *CyO; dSREBP* ^{189} */TM6B, Tb Hu e* stocks. Similar stocks were generated using the P{*w*$^{+}$, *UAS-dSREBP*} responder transgene. For rescue experiments, the driver and responder lines were crossed and the emergence of various classes of adults was scored using the *Hu* and *Cy* markers.

Analysis of fatty acid composition
Embryos were collected and plated on 60 mm dishes containing 9 ml of semidefined medium (Backhaus et al., 1984). Larvae of the desired genotype were collected from plates between 37-41 hr after egg laying. 400-450 larvae were pooled for each sample and three samples were analyzed for each genotype. The larvae were homogenized in 200 μl Buffer A supplemented with a cocktail of protease inhibitors. 150 μl of the lysates were extracted with Folch reagent (2:1 chloroform:methanol) (Folch et al., 1957), after the addition of 40 μg of pentadecanoic acid (C15:0) as an internal standard. Samples were transesterified according to the method of Lepage and Roy (Lepage and Roy, 1986). Fatty acid methyl esters were separated by gas chromatography using a Hewlett Packard 6890 Series GC System. The identity of the fatty acid methyl esters was determined by comparing retention times with 37 methylated fatty acid standards (Supelco 37 Component FAME Mix). Fatty acids in each sample were quantified by comparison to pentadecanoic acid. Pentadecanoic acid was not detected in samples processed without this addition. The remaining 50 μl of each homogenate was centrifuged at 20,000 g for 10 min. Protein was measured from the supernatants using a BCA protein assay kit (Pierce).

Nutritional rescue of *dSREBP* mutants
Preparation of medium
The relevant compound was added in a solid form (w/vol), with constant stirring, to molten cornmeal-molasses-agar and aliquoted into vials at 9 ml/vial, stored at 4ºC and used within one week. Soybean lipid extract was purchased from Avanti Polar Lipids. Na C12:0, Na C14:0, Na C16:0, Na C18:0, Na C18:1 and tripalmitin were purchased from Sigma Aldrich. Na C16:1 was prepared from C16:1 (Sigma Aldrich) as described (Hannah et al., 2000).
Rescue of mutants
Embryos were collected overnight from a *dSREBP* ^{189}*/TM3,Act-GFP,Ser* stock. We added 1 mg embryos per vial except as noted in the Table legends. Flies were allowed to develop and emerging adults were scored until they stopped emerging (approximately day 18 after plating). In order to calculate the percent rescue, the observed ratio of homozygotes to heterozygotes was divided by the expected ratio (0.5).

Quantitative analysis of transcripts
Embryos were collected for 2 hr and plated at 20 mg/plate. Larvae were allowed to develop 37–41 hr. Total RNA was prepared from approximately 100 first instar larvae for each genotype examined using RNA-Stat 60 (Tel-Test, Inc) according to the manufacturer's instructions. cDNA was prepared by using the Superscript First-Strand Synthesis kit (Invitrogen). Real-time quantitative TaqMan PCR analysis (Heid et al., 1996) was performed using primers as described previously (Dobrosotskaya et al., 2002), except that 20 ng of cDNA was used per reaction and primers for *CG6295* were (5′-ATC TCTGGCTCGCACTTCAAC, 5′-GGAGGACCAGCCGTGGATA). Expression dRP49 of (5′ – CCCACCGGATTCAAGAAGTTC, 5′ -AAACGCGGTTCTGCAT GAG) was used as an internal standard for normalization. The relative amounts of all mRNAs were calculated using the Comparative C_T method and standard deviation of $\Delta\Delta C_T$ and the range were calculated as described in User Bulletin #2 (PE Applied Biosystems).

Microscopy
Fluorescence images were obtained using a Leica MZ16FA fluorescence microscope equipped with an Evolution MP digital camera (Media Cybernetics) and In Focus software (Meyer Instruments, Houston, TX). GFP fluorescence was visualized using a GFP2(+) filter set for MZ16 FA, 480/40, 510 nm and images were captured using ImagePro software.

Supplemental data

Supplemental data include five figures, five tables, and Supplemental References and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/6/439/DC1/.

Acknowledgments

We are grateful to Ernst Wimmer for *SREBP*^{52} flies, to Edward Tambe-Ebot, Joe Lockridge, and Phuong Pham for excellent technical support, and to Jeff Cormier for real-time PCR analysis. We thank our colleagues Bilal Amarneh, Carol Lah, and Irina Dobrosotskaya for help with plasmid construction, Young Ah Moon and Scott Clark for help with lipid analysis, and Mike Brown and Joe Goldstein for support and encouragement. This work was supported by grants from the American Heart Association (AHA 0130010N), the National Institutes of Health (R01 GM07145701A1), and the Perot Family Foundation.

Received: October 28, 2005
Revised: February 10, 2006
Accepted: April 25, 2006
Published: June 6, 2006

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